Full Paper

Voltammetric and DFT Studies on Viloxazine: Analytical Application to Pharmaceuticals and Biological Fluids

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Abstract
The oxidative behavior of viloxazine was studied at a glassy carbon electrode in different buffer systems using cyclic, differential pulse and square-wave voltammetry. The oxidation process was shown to be diffusion-controlled and irreversible over the studied pH. The voltammetric study of the model compounds, 2-ethoxyanisole and morpholine, associated with quantum mechanical (DFT) calculations, allowed to elucidate the oxidation mechanism of viloxazine. An analytical method was developed for the quantification of viloxazine using an acetate pH 5 buffer solution as a supporting electrolyte. A linear response was obtained in the range 7 to 45 μM, with a detection limit of 0.8 μM. Validation parameters such as sensitivity, precision and accuracy were evaluated. The proposed method was successfully applied to the determination of viloxazine in pharmaceutical formulations and in human serum. The results were statistically compared with those obtained through an established high-performance liquid chromatography technique, no significant differences having been found between the two methods.

Keywords: Viloxazine, Square-wave voltammetry, Oxidation, DFT calculations, Pharmaceutics, Biological fluids

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1. Introduction

Depression is becoming a quite common psychological disorder, affecting millions of people worldwide. The first drugs used to treat depression were tricyclic antidepressants, such as amitriptyline and imipramine, as well as the monoamine oxidase inhibitors. The side-effects, toxicity and severe drug-drug interactions of these types of compounds lead to the introduction of new antidepressants, in the light of the remarkable advances in the understanding of the central nervous system (CNS). Nowadays, an important group of such drugs comprises the selective noradrenalin reuptake inhibitors, including viloxazine.

Viloxazine, 2-[(2-ethoxyphenoxy)methyl] morpholine (VX, Fig. 1), is a member of a series of compounds which were synthesized in order to exploit the central nervous system activity of the aryloxypropanolamine type of β-adrenoreceptor antagonist. Viloxazine is indicated for use in the treatment of all types of depression. It may be particularly useful in the enderly when they have a tendency to delirium, as long as they have no history of proneness to nausea. In contrast to other antidepressants (e.g., imipramine), viloxazine has minimal sedative anticholinergic or adrenergic effects in humans and it acts partly as an amphetamine-type CNS stimulant, although there is little evidence of dependence or non-durability of drug effects [1].

Viloxazine is rapidly and almost completely absorbed following oral administration. The unchanged drug is the major component found in blood at all times and it is probably the pharmacologically active agent. It has a short plasma half-life and is extensively metabolized and rapidly excreted through the kidneys [1].

Analytical methods for the detection of antidepressants are not only of interest in the field of clinical toxicology but also in forensics, as this type of compounds is often involved in intoxication events [2–4]. The development of new quantitative techniques for drug assessment is thus of the utmost importance. These may also be used for monitoring patient compliance and to establish relationships between blood concentration and the therapeutic effects, which are not always fully understood. Moreover, therapeutic drug monitoring can provide valuable information for attaining a rational and cost-effective use of psychiatric drugs [4–6].

Electroanalytical methods have long been used for the determination of a wide range of drug compounds due to their simplicity, low cost and relatively short analysis time when compared to other techniques. Furthermore, the knowledge of the electrochemical properties of a drug is an

important pharmaceutical tool since it can be relevant to understand its metabolic fate or in vivo redox processes and pharmacological activity [7–9].

Very few studies are to be found in the literature on the quantification of viloxazine, most of these relying on chromatographic methods [10–13]. To this date, no information on the electrochemical redox properties of this drug has been reported, and no analytical method has been proposed for its determination in pharmaceutical preparations or in human fluids.

The purpose of the present study is to carry out a detailed investigation on the electrochemical behavior of viloxazine at a glassy carbon electrode using cyclic, differential pulse and square-wave voltammetry. In order to fully elucidate its oxidation mechanism, the voltammetric study of the model compounds 2-ethoxyanisole (EA) and morpholine (MP) (Fig. 1) was also undertaken. Also, in an effort to further clarify the electronic properties of the compounds under study, quantum mechanical calculations, at the density functional theory (DFT) level, were performed. As a final goal, a simple, rapid, sensitive and reliable electroanalytical procedure is proposed for the determination of viloxazine in pharmaceutical dosage forms and in human blood serum.

2. Experimental

2.1. Apparatus

Voltammetric experiments were performed using an Autolab PGSTAT 12 potentiostat/galvanostat (EcoChemie, Netherlands) associated to an one-compartment glass electrochemical cell equipped with a three-electrode system consisting of a glassy carbon working electrode (GCE) ($d =$ 2 mm), a platinum wire counter electrode and an Ag/AgCl saturated KCl reference electrode. The glassy carbon working electrode was polished manually with aqueous slurry of alumina powder (BDH) on a microcloth pad and rinsed with water before use. All measurements were made at room temperature.

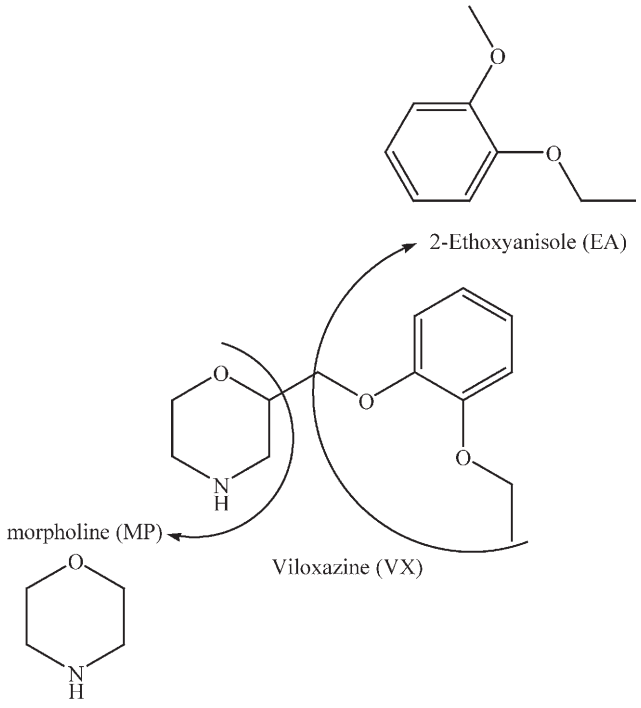


Fig. 1. Chemical formulae of viloxazine, 2-ethoxyanisole and morpholine.

The optimum instrumental parameters to be used in the square-wave voltammetric (SWV) quantification were chosen by studying the variation of the peak current (I_p) with the square-wave frequency (f), pulse amplitude (E_s) and ionic strength (I). The system was optimized, particularly in respect of maximum peak current and reproducibility.

A Crison pH-meter with glass electrode was used for the pH measurements (Crison, Barcelona, Spain).

The HPLC method [11] was performed using a HPLC/DAD system consisting in a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a commercially prepacked Nucleosil RP-18 analytical column (250 mm × 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany) and UV detection (Jasco model 875-UV) at the maximum wavelength determined by the analysis of the UV spectrum (220 nm). The mobile phase was 20 mM phosphate buffer pH 3.8 – acetonitrile, at a flow rate of 1.0 mL per minute at room temperature. The acetonitrile content of the mobile phase was increased linearly from 25% to 40% during the first 10 min and then to 44% in 8 min.

The chromatographic data was processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

2.2. Chemicals and Standards

Viloxazine hydrochloride was provided by AstraZeneca (Queluz, Portugal). Morpholine and 2-ethoxyanisole were purchased from Sigma-Aldrich Química (Sintra, Portugal). Analytical grade reagents purchased from Merck (Darmstadt, Germany) were used without additional purification. Vivalan, labelled to contain 100 mg viloxazine per tablet, was a gift from AstraZeneca.

Deionized water (conductivity < 0.1 μS cm^{-1}) was used throughout the experiments. Buffer solutions employed for voltammetric determinations were 0.2 M in the pH range 1.2–12.2.

HPLC-grade acetonitrile was obtained from Merck. Phosphate buffer 20 mM was prepared in deionized water and the pH was adjusted to 3.8 with 10% *ortho*-phosphoric acid. Prior to use, the solvents were filtered through a 0.45-μm filter.

A standard stock solution of viloxazine (2 mM) was prepared in deionized water. In a typical run, 10 mL of supporting electrolyte was transferred to the electrochemical cell and the required volume of the standard stock

solution of viloxazine was added. For calibration curves, standard solutions were prepared in the voltammetric cell, adding accurate volumes of the stock standard solution to the selected acetate pH 5 supporting electrolyte in order to obtain concentrations between 7 and 45 μM. The calibration curve for SWV analysis was constructed by plotting the peak current against the viloxazine concentration (7.4, 14.8, 22.0, 29.1, 36.1 and 43.1 μM). The limit of quantification (LOQ) and the limit of detection (LOD) were calculated according to USP guidelines [14]. A S/N ratio of ten and three was used respectively. Method precision was checked at different days, within day ($n=5$) and between days ($n=5$) for three different concentrations. The accuracy of the proposed method was determined by comparing the results obtained with those from a previously published HPLC method [14]. The precision and accuracy of the analytical method was also described by the use of relative errors (Bias%).

2.3. Analysis of Pharmaceutical Dosage Form

For viloxazine determination in pharmaceuticals, an amount of ten tablets were weighed and ground to a homogeneous fine powder in a mortar. An accurately weighed portion equivalent to a stock solution of a concentration about 2 mM was transferred to a volumetric flask and dissolved with deionized water. The mixture was sonicated for 5 min to effect complete dissolution and filtered to remove any remaining insoluble matter. Working solutions of the pharmaceutical formulation were prepared exactly as the standard solutions.

2.4. Analysis of Spiked Serum Samples

Human serum samples were collected from healthy volunteers and stored frozen until the assay. An aliquot volume of sample was fortified with viloxazine and dissolved in deionized water to achieve final concentration of 2 mM. The mixture was treated with 0.7 mL of methanol as serum denaturating and precipitating agent, and then the volume was completed to 2 mL with the same serum sample. The tubes were vortexed for 5 min and then centrifuged for 10 min at 4000 rpm for removing of protein residues. The supernatant was taken carefully. Appropriate volumes of this solution were added to acetate pH 5 supporting electrolyte and the voltammograms were then recorded.

The concentration of viloxazine in the human serum samples varied in the range of 10 to 45 μM. For the recovery studies the amount of viloxazine in spiked human serum samples was calculated from the related calibration equation.

Methanol and acetonitrile, in different amounts, were tested as serum protein precipitating agents. Yet, the best results were obtained using 0.7 mL of methanol.

2.5. Quantum Mechanical Calculations

The quantum mechanical calculations, geometry optimization and calculation of the harmonic vibrational frequencies, were performed using the GAUSSIAN 03W program [15], within the Density Functional Theory (DFT) approach in order to properly represent the electron correlation effects. The B3LYP hybrid method, which includes a mixture of HF and DFT exchange terms and the gradient-corrected correlation functional of Lee, Yang and Parr [16, 17] as proposed and parameterized by Becke [18, 19], was used, along with the double-zeta split valence basis set 6-31G* [20, 21].

Molecular geometries were fully optimized by the Berny algorithm, using redundant internal coordinates [22]: The bond lengths to within ca. 0.1 pm and the bond angles to within ca. 0.1°. The final root-mean-square (rms) gradients were always less than 3×10^{-4} hartree bohr^{-1} or hartree radian^{-1}. No geometrical constraints were imposed on the molecules under study.

Atomic charges were also calculated and a population analysis was undertaken, yielding the highest occupied and lowest unoccupied molecular orbitals for the systems studied (HOMO and LUMO, respectively).

3. Results and Discussion

3.1. Voltammetric Studies

No electrochemical data is to be found in the literature concerning the electrode behavior of viloxazine. The voltammetric study presently reported, using different electrochemical techniques, was therefore performed in a broad pH range (1.2 – 12.2), using a glassy carbon electrode.

The differential pulse voltammetric (DPV) behavior of viloxazine yielded one anodic wave, $E_p=+1.20$ V, starting at pH 1 (Fig. 2), as a result of an oxidation process occurring on the aromatic nucleus of the molecule. As the pH increase, a second poorly resolved shoulder appears, $E_p=+1.10$ V near pH 7 (Fig. 2a), corresponding to the oxidation of the secondary amine group of the morpholine moiety. The peak potential (E_p) of the main oxidation process does not depend on the solution pH, since no significant variation was observed (Fig. 2b).

To better comprehend the oxidative behavior of viloxazine, the voltammetric assessment was extended to the study of the model compounds 2-ethoxyanisole and morpholine (Fig. 1).

The DPV behavior of 2-ethoxyanisole yielded one anodic wave, $E_p=+1.15$ V, starting at pH 1 (Fig. 3a). The peak potentials (E_p) obtained does not depend on the solution pH, since no significant variation was observed upon pH variation. At some acidic pH's a second poorly resolved wave is seen at $E_p=+1.40$ V. This oxidative pattern closely matches the behavior observed for viloxazine, in particular considering its main oxidation process (Fig. 4).

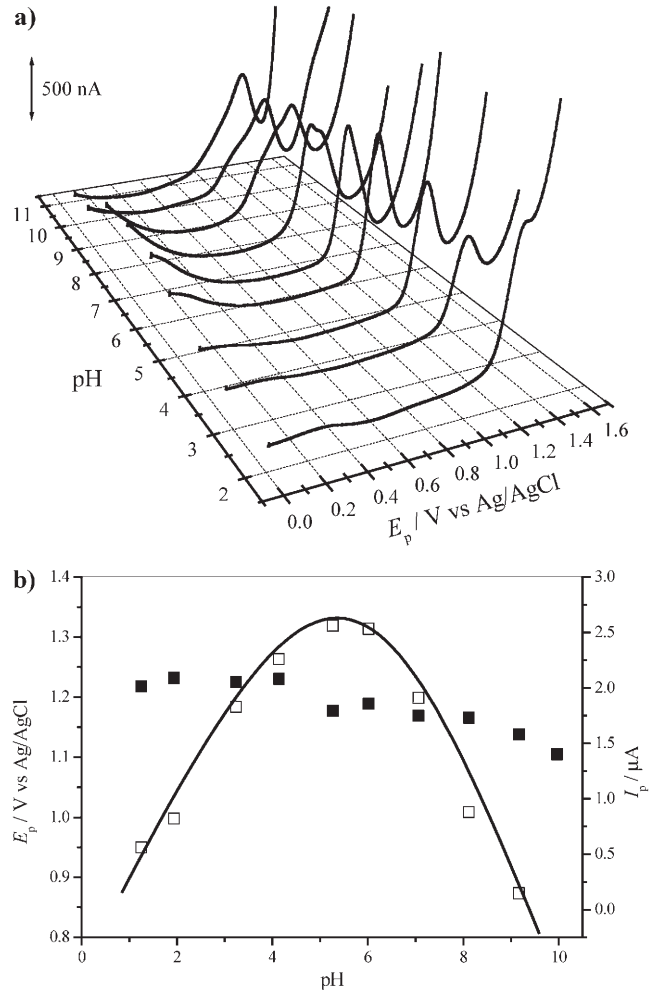


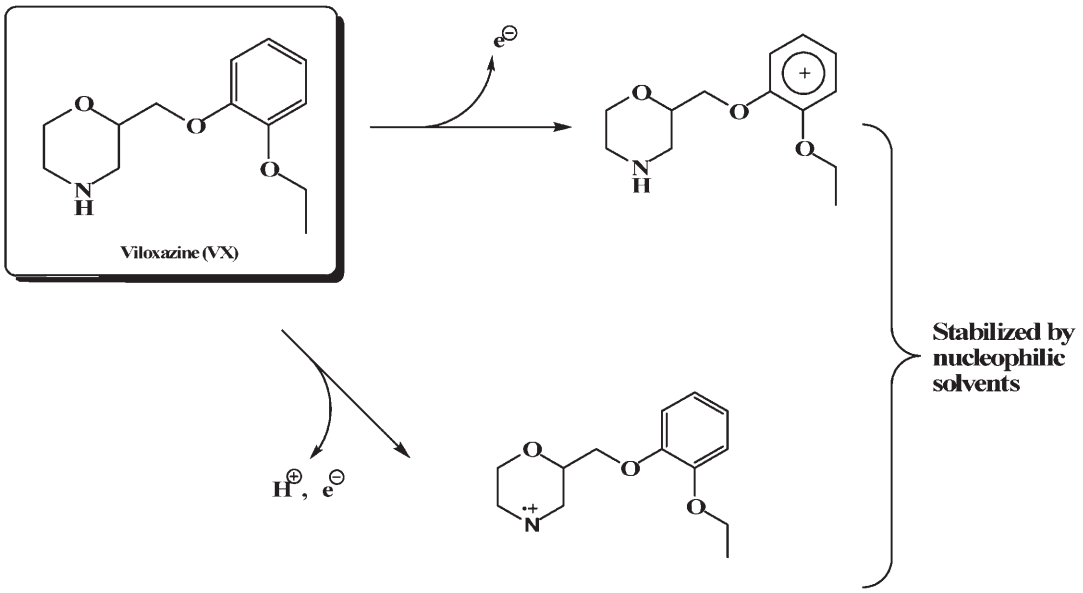
Fig. 2. a) 3D plot and b) plots of E_p (filled symbols) and I_p (open symbols) vs. pH from differential pulse voltammograms of 0.2 mM solutions of viloxazine in different buffer electrolytes as a function of pH. Scan rate 5 mV s^{-1}.

These results are consistent with the data previously reported on alkoxylbenzenes [23–25], which indicates that oxidation proceeds via formation of a cation-radical intermediate. Thus, it seems reasonable to suppose that viloxazine oxidation occurs at the aromatic nucleus, through a one-electron transfer, yielding an unstable cation-radical (Scheme 1). The second oxidation wave observed at acidic pH's for 2-ethoxyanisole, not detected for viloxazine, must then be related to the oxidation of the species resulting from the formation and dimerization of the initial cation radical [24, 25].

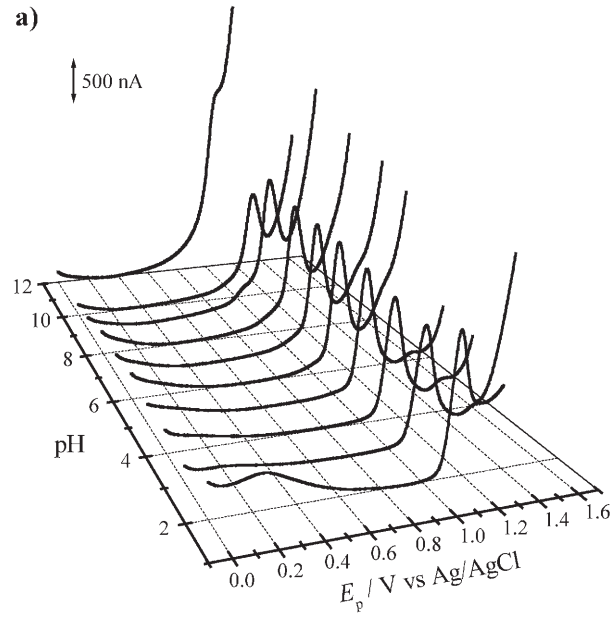
The differential pulse voltammetric study of morpholine yielded an anodic shoulder, $E_p = +0.98$ V, starting at pH 8 (Fig. 3b). The appearance of this peak is connected with the acid-base properties of morpholine ($pK_a = 8.4$). In fact, at this pH deprotonation of the amine group occurs and, as a result, an oxidative process can take place (due to the existence of an electron lone pair). Once more, these results allow to validate the data obtained for viloxazine that has a similar pKa ($pK_a = 8.13$) (Fig. 4). Hence, it may be proposed that the second anodic process occurring in viloxazine is related to the oxidation of the secondary amine group of the heterocyclic nucleus (Scheme 1).

Regarding the analytical application of this type of studies, the influence of the pH on the viloxazine peak current at a glassy carbon electrode was also investigated, using DPV. The plot of I_p vs. pH indicates that the peak current reaches a maximum for pH 5 (Fig. 2b). The experimental results also showed that the curve shapes are better defined in pH 5 acetate buffer as compared to Britton–Robinson buffers. Accordingly, this buffer was chosen as the best supporting electrolyte and was used throughout the analytical study.

Cyclic voltammetric measurements performed for 0.2 mM viloxazine solutions evidenced the irreversible



Scheme 1.



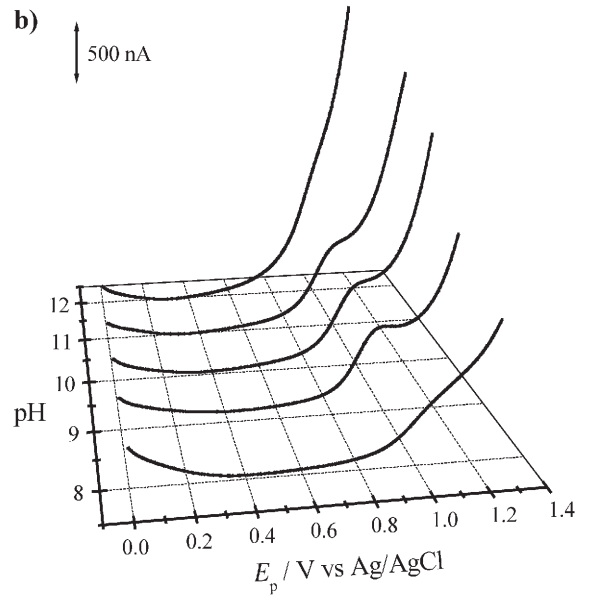


Fig. 3. 3D plot from differential pulse voltammograms of 0.2 mM solutions of a) 2-ethoxyanisole and b) morpholine in different buffer electrolytes as a function of pH. Scan rate 5 mV s^{-1}.

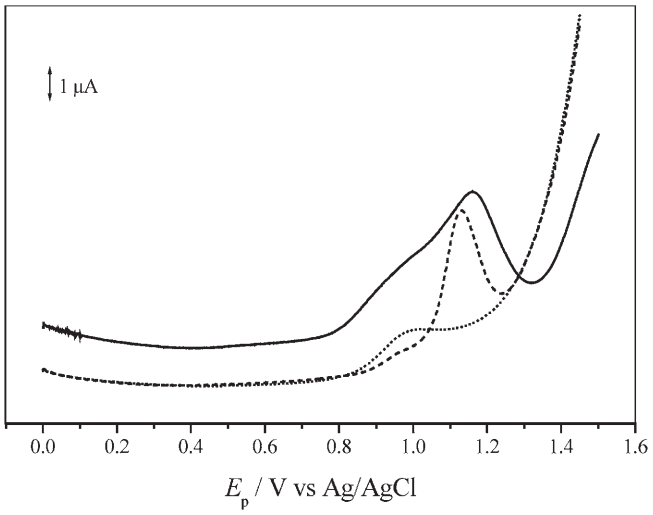


Fig. 4. Differential pulse voltammograms of a 0.2 mM solutions of (—) viloxazine, (- - -) 2-ethoxyanisole and (····) morpholine in pH 9 buffer electrolyte. Scan rate 5 mV s^{-1}.

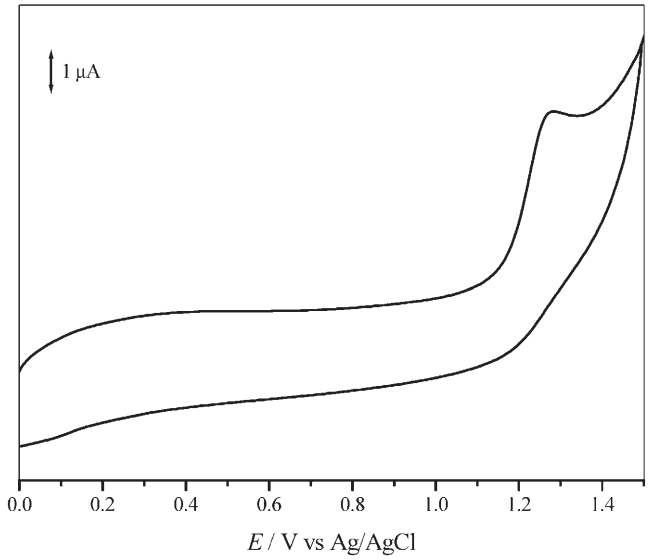


Fig. 5. Cyclic voltammogram for 0.2 mM solution of viloxazine in pH 5 acetate buffer. Scan rate 100 mV s^{-1}.

nature of the oxidation process at the glassy carbon electrode, as no peaks were observed in the reverse scans (Fig. 5). The irreversibility was also studied through a Tafel treatment of voltammetric curves, which yielded a αn value of 0.41, indicating the irreversibility of the electron-transfer process [26].

The study of the influence of the scan rate from 0.01 to 0.5 V s^{-1} for the viloxazine oxidation allowed to conclude that the peak current (I_p) changes linearly with the scan rate (v), according to the equation $I_p = Av^x$. The regression of log I_p vs. log v yielded a line of slope 0.48, typical of a diffusion-controlled process [27]. Increasing the scan rate, the peak potential shifted towards more positive values, as expected for an irreversible oxidation reaction. These studies reveal that no important electrode fouling and surface deactivation effects occur under the experimental conditions used.

3.2. DFT Calculations

Quantum mechanical (DFT) calculations were performed for morpholine (MP), *N*-protonated morpholine (MPH), 2-ethoxyanisole (EA), viloxazine (VX) and *N*-protonated viloxazine (VXH), in order to complement the results obtained by electrochemical methods, thus providing relevant information on the viloxazine oxidation mechanism. In

fact, the determination of the structural characteristics and the electronic-density distribution within a molecule is valuable for the understanding of its electrochemical behavior.

The lowest energy optimized geometries calculated for viloxazine, 2-ethoxyanisole and morpholine are represented in Figure 6, and the corresponding and most significant Mulliken atomic charges are depicted in Table 1. The analysis of charge populations evidence that the nitrogen from the morpholine moiety of viloxazine, as well as atoms C_{11} to C_{14} from the aromatic ring (Fig. 6) have a higher electron density than the rest of the molecule, thus confirming their vital role in the oxidation process of viloxazine.

Apart from an accurate determination of the ground state geometry of viloxazine, the nature of its frontier molecular orbitals (HOMO and LUMO) was also studied, since these are directly involved in the reactivity (electrophilicity/nucleophilicity) of the molecule, thus ruling its electrochemical behavior. Actually, electrochemical reactions can be achieved by a direct electron transfer, at a particular potential value. If electrons are transferred from the HOMO of a compound, a correlation is expected to exist between the E_{HOMO} and the oxidation potential, because, according to Koopmans' theorem, the negatives of the orbital energies in the ground state are equal to the ionization potentials. Thus, it may be assumed that the oxidative processes undergone by a certain system are driven by the electron density profile of its HOMO's.

Table 1. Atomic Mulliken charges calculated (B3LYP/6-31G*) for morpholine (MP), *N*-protonated morpholine (MPH), 2-ethoxyanisole (EA), viloxazine (VX) and *N*-protonated viloxazine (VXH).

Atom [a]	MP	MPH	EA	VX	VXH
O_2	−0.49	−0.46	–	−0.50	−0.49
N_5	−0.61	−0.48	–	−0.56	−0.26
C_{11}			−0.20	−0.20	
C_{12}			−0.14	−0.14	
C_{13}			−0.14	−0.14	
C_{14}			−0.20	−0.20	
$\Sigma\ C_{11}$–C_{14}			−0.68	−0.68	
C_{15}			0.33	0.34	
C_{16}			0.34	0.34	
O_{21}			−0.50	−0.52	
O_{22}			−0.51	−0.51	

[a] Atoms are numbered according to Figure 6.

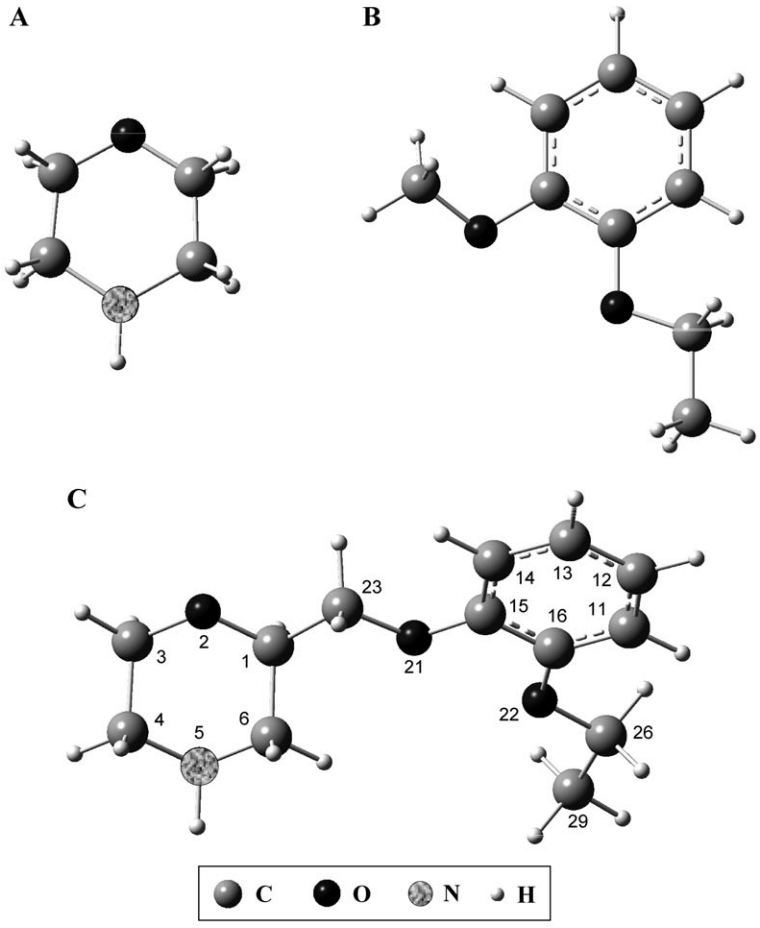


Fig. 6. Schematic representation of the most stable calculated geometries for morpholine (A), 2-ethoxyanisole (B), and viloxazine (C). B3LYP/6-31G* level of calculation. The atom numbering for viloxazine is included.

The population analysis presently carried out for viloxazine at the DFT level showed that in the protonated molecule the HOMO and HOMO-1 (highest occupied and second highest occupied molecular orbitals, respectively) are localized on the carbons of the aromatic ring (Fig. 7a). For unprotonated viloxazine, in turn, while HOMO is still centered on the aromatic ring, HOMO-1 is delocalized to the morpholine moiety (Fig. 7b). These results corroborate the data yielded by the voltammetric techniques, which allows to conclude that the anodic oxidative processes occurring for viloxazine are mainly due to a one-electron transfer at the aromatic nucleus coupled to the oxidation of the secondary amine group from the morpholine moiety.

3.3. Analytical Applications

On the basis of the voltammetric behavior of viloxazine, a quantitative analytical method was developed to determine the drug content in different types of samples. To select the best electrochemical method for this purpose, the anodic peaks obtained by differential pulse and square-wave voltammetry (SWV) were compared. SWV was found to be the faster method, gives the best ratio of peak-to-background current and provide sharper and better defined peaks, leading to an enhanced resolution.

Optimization of the SWV parameters regarding peak definition and current was accomplished using $f = 175$ Hz, $E_s = 50$ mV and $I = 0.2$ M (see Sec. 2).

Under the described experimental parameters, linear calibration plots were obtained for viloxazine in the concentration range 7 to 45 μM. Analytical data for the calibration graphs are summarised in Table 2. The limit of detection (LOD) and the limit of quantification (LOQ), obtained after five runs, are also presented in Table 2.

The precision of the method was evaluated by repeatedly ($n = 5$) measuring viloxazine, at three levels of concentration (10, 25 and 40 μM) within a day and over five consecutive days (Table 2).

3.4. Determination of Viloxazine in Tablets

In order to evaluate the applicability of the proposed method, a commercial sample containing viloxazine (Vivalan) was analyzed. Furthermore, the reliability of this method was tested by analyzing Vivalan through a previously reported HPLC technique [11].

For the SWV and HPLC methods, five different independent measurements were carried out. These results, which summarised in Table 3, show that SWV method yields an average concentration which is in good agreement with the label claim (100 mg/tablet). Moreover, values obtained by SWV fitted reasonably well with those yielded by chromatography.

The F- and t-test were carried out on the data and statistically examined the validity of the obtained results. For a confidence level of 95%, the values of t- and F-tests

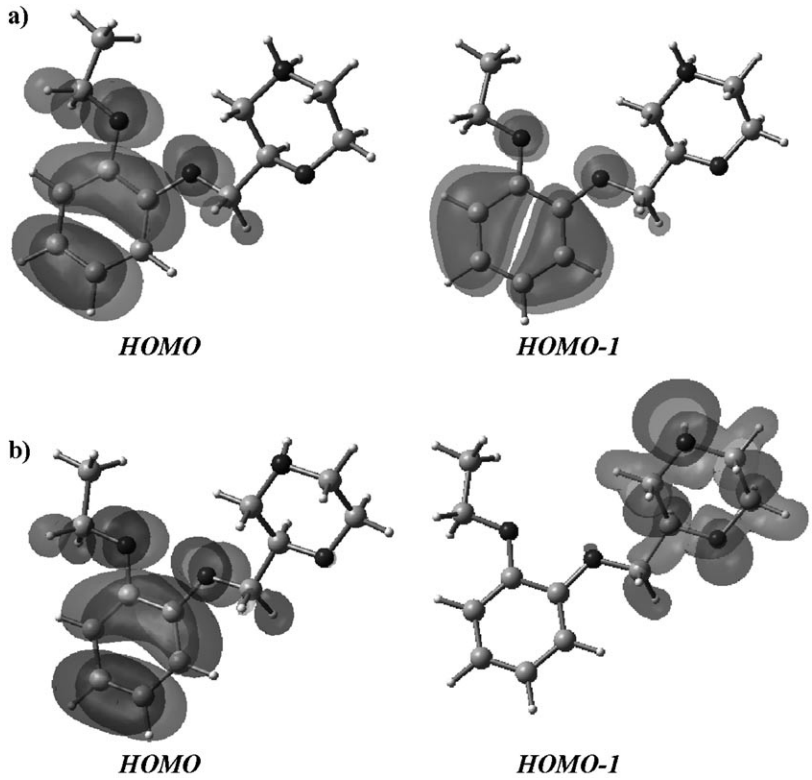


Fig. 7. Electronic structures calculated (B3LYP/6-31G*) for the a) *N*-protonated viloxazine and b) viloxazine molecule highest occupied molecular orbital (HOMO) and second highest occupied molecular orbital (HOMO-1).

Table 2. Linearity range, quantification and detection limits and intra- and interday repeatability for determination of viloxazine, using the square-wave voltammetric method (SWV), in supporting electrolyte and human serum samples.

	SWV	
	Supporting electrolyte	Serum
Linearity range (μM)	7 – 45	10 – 45
Calibration graph ($n = 6$)		
Intercept (μA)	0.013	0.025
Slope (mA μM^{-1})	49.6	50.9
Correlation coefficient	0.998	0.998
Standard error of intercept	0.021	0.018
Standard error of slope	1.16	1.35
Intraday precision ($n = 5$) [a]		
10 μM	2.8	2.3
25 μM	1.6	1.8
40 μM	2.3	2.5
Interday precision ($n = 5$) [a]		
10 μM	2.6	2.8
25 μM	2.1	1.9
40 μM	1.8	2.2
LOD (μM)	0.8	1.6
LOQ (μM)	3.2	6.3

[a] Precision as coefficient of variation (CV, %) = standard deviation divided by mean measured concentration × 100.

were less than those of theoretical values, showing that there is no significant difference between the proposed SWV technique and the HPLC comparative method.

In order to verify whether the excipients from the pharmaceutical formulation, such as starch, calcium phosphate, cellulose, sodium glycolate, or glycerine, interfere in any way with the method of analysis, recovery studies were carried out after the addition of known amounts of the pure drug to the viloxazine formulation. It was verified that the excipients present in the tablets do not significantly affect the analysis (Table 3).

The results presently obtained demonstrate the validity of the proposed method for an accurate determination of viloxazine in tablets. Furthermore, these results also revealed that both methodologies applied had a comparable precision and accuracy.

3.5. Determination of Viloxazine in Serum Samples

In order to examine the possibility of applying the newly proposed analytical method to biological samples, the quantification of viloxazine in human serum was also tested. Calibration equation parameters and validation data are summarized in Table 2. Serum samples were spiked with viloxazine in order to achieve final concentrations of 10, 25 and 40 μM.

Square-wave voltammograms obtained from the determination of viloxazine in human serum samples are presented in Figure 8. The recoveries obtained were generally good and are shown in Table 4.

Table 3. Square-wave voltammetric method (SWV), HPLC comparative method assay results from viloxazine tablets (Vivalan) and mean recoveries in spiked tablets.

	SWV	HPLC
Labeled claim (mg)	100	100
Amount found [a] (mg)	99.2	99.8
RSD (%)	1.9	1.3
Bias (%)	0.8	0.2
t [b]	0.6	
F [c]	2.1	
Amount added (mg)	20.00	
Amount found [a] (mg)	20.09	
Recovery (%)	100.4	
RSD (%)	1.6	
Bias (%)	−0.45	

[a] Mean of five experiments.
[b] Tabulated *t*-value for $P = 0.05$ and eight degrees of freedom is 2.31.
[c] Tabulated *F*-value for $P = 0.05$ and $f_1 = f_2 = 4$ is 6.39.

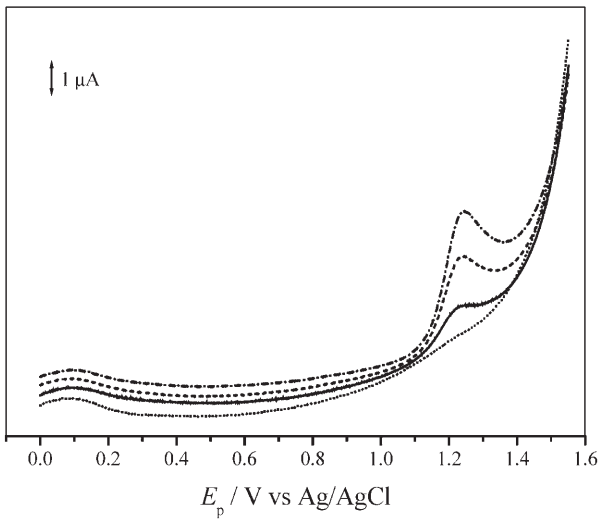


Fig. 8. Differential pulse voltammograms obtained from the determination of viloxazine in spiked human serum samples. (···) Blank serum; (—) 10 μM; (**---**) 25 μM and (– • –) 40 μM viloxazine in human serum samples.

4. Conclusions

The electrochemical oxidation process of viloxazine on a glassy carbon electrode was studied. The voltammetric and quantum mechanical analysis performed for viloxazine, as well as for the model compounds 2-ethoxyanisole and morpholine, evidenced that the oxidative mechanisms occurring within these systems are driven by the charge localization of HOMO and HOMO-1 frontier molecular orbitals, located on the aromatic ring and morpholine moiety.

This study enabled the development and proposal of a voltammetric technique suitable for the determination of viloxazine in both pharmaceutical formulations and biological samples. In fact, the results presently gathered showed that the proposed method is both selective and sensitive, and does not lead to statistically significant differences when

Table 4. Results obtained for viloxazine analysis from spiked human plasma using the square-wave voltammetric method (SWV).

Viloxazine added (μM)	Viloxazine found (μM)	Average recovery [a] (%)	*RSD* (%)	Bias (%)
10	10.03	100.3	1.2	−0.3
25	25.12	100.4	1.1	−0.5
40	39.78	99.5	0.8	0.6

[a] Mean of three determinations.

compared to an established HPLC methodology. The main advantages of this new methodology is that it may be directly applied to the analysis of pharmaceutical dosage forms and biological samples without any special treatment of the sample (such as separation procedures), since there is no interference from excipients or other endogenous substances.

Moreover, the reported results outlook the possibility of using this technique for monitoring viloxazine for pharmacokinetic and pharmacodynamic purposes.

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6. References

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